Functional complementation of the *Schizosaccharomyces pombe wis1* mutant by *Arabidopsis MEK1* and non-catalytic enhancement by *CTR1*

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Abstract ***Arabidopsis thaliana MEK1*** **encodes a MAPKK homolog whose role in plants is currently unknown. High (but not low) expression of** ***MEK1*** **rescued the Δ*wis1* (MAPKK) mutant of the *Schizosaccharomyces pombe* Win1/Wis4-Wis1-Sty1 stress-activated MAPK pathway. Rescue was dependent upon upstream and downstream components of the pathway, suggesting that** ***MEK1*** **might function in a homologous MAPK pathway in plants. When** ***MEK1*** **was expressed at a low level, rescue of Δ*wis1* was achieved by co-expressing *Arabidopsis CTR1* (a putative MAPKK kinase (MAPKKK)). CTR1 constructs alone did not rescue the pathway, indicating that CTR1 augmented MEK1 function. Further data indicated that this enhancement was not due to CTR1 kinase activity.**

Key words: Mitogen-activated protein kinase; Stress-activated MAPK pathway; *trans*-complementation; *Arabidopsis thaliana*; *Schizosaccharomyces pombe*

1. Introduction

The MAPK pathway is a conserved eukaryotic signaling module that controls numerous cellular activities in response to receptor signals [1]. A MAPKKK activates a specific MAPKK by phosphorylation on serine and threonine residues, and MAPKK in turn activates a specific MAPK by phosphorylation on threonine and tyrosine residues. Appropriate cellular responses are then regulated by the MAPK through activation of elements in the nucleus and cytoplasm. Different MAPK pathways can integrate a variety of signals through interactions with other protein kinases or G proteins, which are ultimately controlled by receptor tyrosine kinases, cytokine receptors or two-component histidine kinase receptors. Examples of the latter are the *Saccharomyces cerevisiae* osmolarity-response pathway [2], the *Schizosaccharomyces pombe* stress-activated pathway [3] and potentially the *Arabidopsis thaliana* ethylene hormone-response pathway [4].

In plants, MAPK pathways have been implicated in responses to stresses, wounding, hormones and pathogen attack [5,6]. Although a number of plant genes encoding MAPKKKs, MAPKKs and MAPKs have been cloned [5,6], the specific roles for most of these are unknown. Although a complete MAPK module has yet to be identified in plants, a putative module involving *Arabidopsis* ATMEKK1 (MAPKKK), MEK1/ATMKK2 (two MAPKKs) and ATMPK4 (MAPK) was recently proposed based on physical and functional associations in yeast [7,8]. *MEK1* was capable of rescuing the Δ*pbs2* (MAPKK) mutant of the *S. cerevisiae* osmolarity-response pathway and this rescue was enhanced by co-expression of an activated form of *ATMEKK1*, suggesting that ATMEKK1 can activate MEK1 [8]. Similarly, co-expression of *MEK1* and *ATMPK4* rescued the *S. cerevisiae* Δ*bck1* (MAPKKK) and Δ*mpk1* (MAPK) mutants, suggesting that MEK1 can activate ATMPK4 [7,8]. It is unclear from these complementation studies whether these MAPK pathway genes are functional homologs of the yeast genes.

Ethylene is a gaseous plant hormone that regulates numerous developmental processes, as well as responses to various stresses [4]. Ethylene signaling is thought to utilize a MAPK pathway based on the finding that a negative regulator of ethylene responses, CTR1, is a putative MAPKKK in *Arabidopsis* [9]. The CTR1 sequence is most similar to the Raf protein kinase family. The C-terminal portion of CTR1 contains all 11 kinase subdomains, including the residues characteristic of serine/threonine specificity [9]. Kinase activity has been detected for CTR1 in vitro [10,11]. CTR1 acts downstream of a family of histidine protein kinase-like ethylene receptors, two of which have been shown to physically interact with the N-terminal non-catalytic domain of CTR1 [12]. The mechanisms by which CTR1 is regulated are currently unknown, as are the immediate downstream targets of CTR1.

To study the regulation of CTR1, we were interested in developing a heterologous system in which we could easily assay CTR1 activity. Toward this aim, we tried, unsuccessfully, to rescue a variety of yeast MAPKKK mutants with various CTR1 constructs (K.L. Clark, W. Ding, Z. Pan and C. Chang, unpublished). This led us to try co-expressing *CTR1* and *Arabidopsis MEK1* [13], with the rationale that the *MEK1* product might serve as a substrate for CTR1. Similar co-expression strategies utilizing yeast MAPK pathway mutants have been demonstrated previously [7,8,14,15]. The pathway we employed is the *S. pombe* stress-activated pathway, which controls the cell cycle, initiation of sexual differentiation and responses to environmental stresses such as heat shock, high osmolarity and oxidative stress [16]. The pathway contains at least two functionally overlapping MAPKKKs called Wis4 (=Wik1=Wak1) [17] and Win1 [18,19]. Both of these phosphorylate the Wis1 MAPKK [20],

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Abbreviations: MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAPK kinase; EMM, Edinburgh minimal medium

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which in turn phosphorylates the Sty1 MAPK (=Spc1=Phh1) [21,22]. The Win1/Wis4-Wis1-Sty1 MAPK module is regulated in part by a two-component system comprised of a pair of histidine kinase sensor proteins (J. Millar, personal communications) and a response regulator called Mcs4 [16,23]. *Arabidopsis* MEK1 has 37% amino acid identity to Wis1.

In this paper, we present genetic evidence that *MEK1* can substitute for *Wis1* in the *S. pombe* stress-activated pathway. These results support the possibility that the MEK1 protein acts in a similar stress-activated MAPK pathway in *Arabidopsis*. We also show that CTR1 augments MEK1 activity in the yeast system, however, we find that this occurs by an indirect mechanism, which does not involve phosphorylation of MEK1 by CTR1.

2. Materials and methods

2.1. Yeast strains, growth and transformation

S. pombe strains used in this study were: JM504 (*wis1::ura4 leu1-32 ura4-D18 h+*) (gift of J. Millar), JM1413 (*win1-1 leu1-32 ura4-D18 ade6-M216 his7.366 h−*) [18], JM1368 (*mcs4::ura4 leu-32 urea-D18 ade6-M216 his7-366 h−*) (gift of J. Millar), JM1160 (*sty1::ura4 leu1-32 ura4-D18 ade6-M216 h−*) [16], JM1059 (*leu-32 ura4-D18 ade6-M216 his7-366 h−*) (gift of J. Millar), ED1009 (*wis1::his1 his1-102 leu1-32 ura4-D18 h−*) (gift of P. Fantes).

The lithium acetate method was used for yeast transformation [24]. Transformants were selected and maintained on Edinburgh minimal medium (EMM) with appropriate amino acids plus 16 μM thiamine. The thiamine was added to the medium to repress expression of the cloned genes from the *nmt1* promoter [25].

2.2. Plasmid constructions

The entire *Arabidopsis MEK1* coding sequence was polymerase chain reaction (PCR)-amplified from a cDNA clone template using the primers 5′-GGAATTCCATATGAACAGAGGAAGCTTA-3′ and 5′-TCCCCCGGGTCAAAGATCTGCGTCGGT-3′. The entire *Arabidopsis CTR1* coding sequence (residues 1–821) was PCR-amplified from a cDNA template using the primers 5′-GGAATTCCATATGGAAATGCCCGGTAGA-3′ and 5′-TCCCCCGGGTTACAAATCCGAGCGGTTGGG-3′. For truncated forms of CTR1 (CTR470, CTR488, CTR503 and CTR551), the forward PCR primers were 5′-TGTCGACATATGGGATTCTCAATGTTTCAT-3′, 5′-TGTCGACATATGGCATTGGCAGAAAATGCTGGT-3′, 5′-TGTCGACATATGCCTCCACAGAACATGATG-3′ and 5′-TGTCGACATATGCTTAATATAAAAGAAAAGAT-3′, respectively. The reverse primer was the same as that used to amplify the full length *CTR1* sequence. The CTR1 N-terminal domain (residues 1–550) was amplified using the same forward primer as used for the full length *CTR1* sequence and the reverse primer was 5′-CCCGGGTTAATCACACCACGGGATGTC-3′. All PCR products were cloned into the pGEM-T vector (Promega) and verified by nucleotide sequencing at the Center for Agricultural Biotechnology Sequencing Facility (College Park, MD, USA). The inserts were then removed with *Nde*I and *Xma*I (or *Sma*I) and ligated into the *Nde*I and *Sma*I sites of *S. pombe* expression vectors pREP1 (or pREP2), pREP41 (or pREP42) and pREP81 (or pREP82) [25,26]. The odd-numbered vectors carry the selectable marker LEU2 (which complements *leu1*) and the even-numbered vectors carry the selectable marker Ura4$^+$. Expression of the inserted genes was under the control of different versions of the thiamine-repressible *nmt1* promoter. The promoter is strongest in pREP1/pREP2, weaker in pREP41/pREP42 and weakest in pREP81/pREP82 [26,27]. *Wis1* gene constructs (pREP1-Wis1, pREP41-Wis1 and pREP81-Wis1) were kindly provided by J. Millar.

A T→A transversion (resulting in Asp-694→Glu) was introduced into the full length *CTR1* cDNA in vector pGEM-T using the Transformer Site-Directed Mutagenesis kit (Clontech) and primer 5′-CAAGGTTTGTGAATTTGGTCTCG-3′. The mutation was confirmed by nucleotide sequencing. The mutated version was subcloned as full length or kinase domain constructs into pREP41 as above.

2.3. Analysis of yeast transformants

For osmolarity stress, colonies were streaked onto YEPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose) containing 1.5 M sorbitol or 0.8 M KCl and incubated for 3 days at 30°C. For temperature stress, colonies were streaked onto YEPD medium and incubated for 3 days at 37°C. To test for lethality, colonies were streaked onto EMM medium with appropriate amino acids and incubated for 3 days at 30°C. Transformations were performed multiple times and five or six independent colonies were analyzed each time.

3. Results

3.1. High expression of Arabidopsis MEK1 rescues the S. pombe Δwis1 mutant

The *S. pombe* Δ*wis1* mutant cannot survive under certain stress conditions, including high osmolarity (e.g. 1.5 M sorbitol or 0.8 M KCl) or a high temperature (e.g. 37°C) [16,17]. To test whether the *Arabidopsis MEK1* gene could rescue Δ*wis1* under osmotic or temperature stress, we expressed the

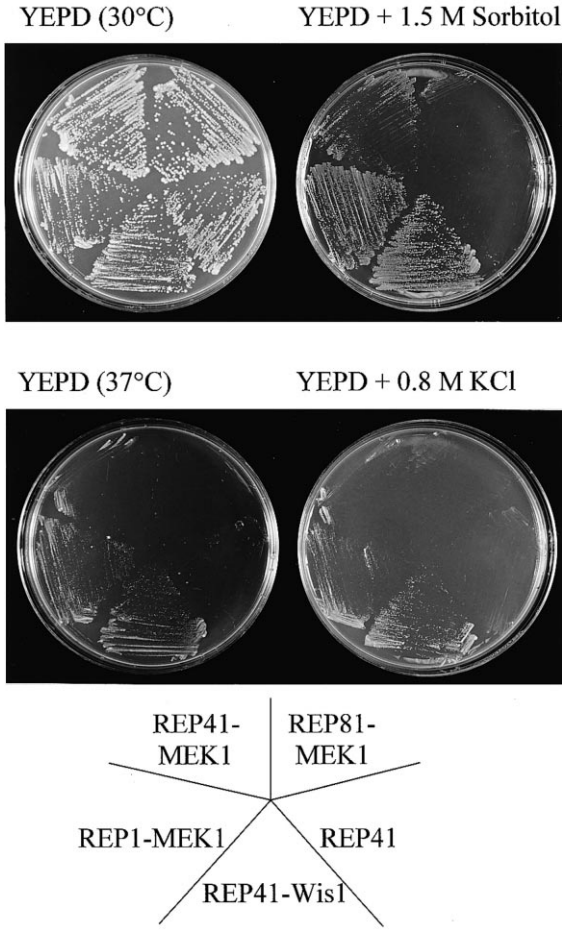

Fig. 1. High expression of *Arabidopsis MEK1* restores growth to the *S. pombe* Δ*wis1* mutant under conditions of high osmolarity or temperature stress. Δ*wis1* cells (JM504) were transformed with the indicated plasmid constructs, which carry progressively weaker promoters. Transformants were streaked onto YEPD medium (with sorbitol or KCl for osmolarity stress) and monitored for growth after 3 days under the conditions shown. As shown, growth of pREP1-MEK1 transformants paralleled that of pREP41-Wis1 control transformants. Weak growth and no growth were obtained for pREP41-MEK1 and pREP81-MEK1 transformants, respectively. No growth was obtained for the pREP41 vector-only transformants.

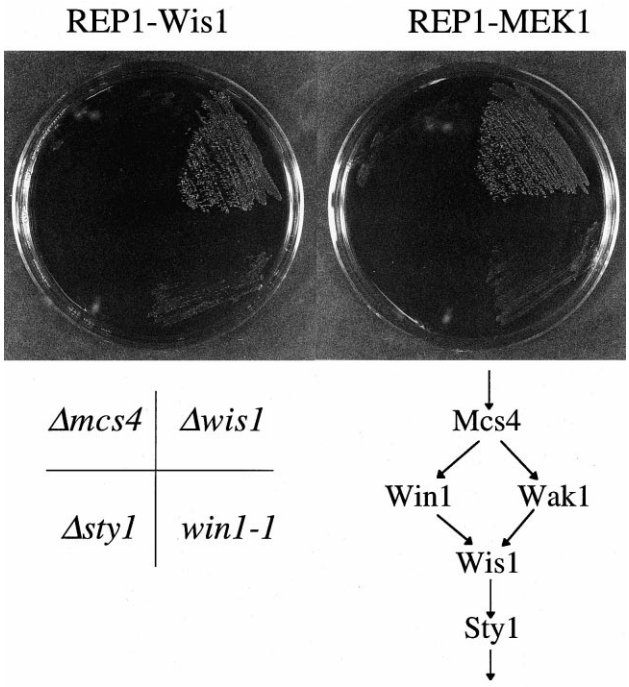

Fig. 2. MEK1 does not suppress other steps of the stress-activated pathway under high osmolarity. pREP1-MEK1 or pREP1-Wis1 were transformed into the indicated mutants Δ*msc4* (JM1368), Δ*wis1* (JM504), Δ*sty1* (JM1160) or *win1-1* (JM1413). All transformants were viable (data not shown). Transformants were streaked onto YEPD medium containing 1.5 M sorbitol and monitored for growth after 3 days at 30°C. As shown, results with pREP1-MEK1 paralleled those with pREP1-Wis1. The partial growth of *win1-1* is seen even in untransformed cells (data not shown) and is thought to be due to there being at least two MAPKKKs in the pathway [18]. A portion of the *S. pombe* stress-response pathway is depicted in the diagram [16].

full length *MEK1* cDNA from three *S. pombe* expression vectors. The series of vectors, pREP1, pREP41 and pREP81, contain progressively weaker versions of the *S. pombe* thiamine-repressible *nmt1* promoter [26,27], thus allowing us to examine the effects of different *MEK1* expression levels. As shown in Fig. 1, Δ*wis1* cells transformed with pREP1-MEK1 (containing the strongest promoter) grew as well as those transformed with the wild-type *S. pombe Wis1* gene (pREP41-Wis1). In contrast, pREP41-MEK1 conferred weak growth and pREP81-MEK1 (containing the weakest promoter) gave essentially no growth (Fig. 1). Control plasmids pREP1-Wis1, pREP41-Wis1 or pREP81-Wis1 rescued Δ*wis1* equally well (data not shown).

3.2. MEK1 activity depends upon upstream activators of Wis1

We next tested whether *MEK1* could rescue other steps of the Msc4-Win1/Wak1-Wis1-Sty1 stress-activated pathway. We found that transformation with pREP1-MEK1 did not restore growth to *S. pombe* Δ*mcs4*, *win1-1* or Δ*sty1* mutants on 1.5 M sorbitol (Fig. 2). This result was identical to that using the *S. pombe Wis1* gene (pREP1-Wis1) (Fig. 2). We did not examine MEK1 activity in the Δ*wak1* mutant, because it can partially grow on 1.5 M sorbitol on its own (due to redundancy with Win1) [16,17]. The inability of *MEK1* to rescue the Δ*mcs4*, *win1-1* or Δ*sty1* mutants suggested that *MEK1* specifically rescued the Wis1 step of the pathway. That is, MEK1 activity required upstream components of the pathway, Win1 and Mcs4, for activation by osmotic stress and acted through the downstream component Sty1.

Under 37°C stress, we found that *MEK1* partially suppressed the upstream *win1-1* mutant (Fig. 3). This result was similar to that obtained using the wild-type *Wis1* gene and was presumably due to a temperature stress-responsive Win1-/Wak1-independent pathway that feeds into Wis1 [18]. Taken together, our results indicated that *MEK1* could functionally replace the *Wis1* gene.

As a final comparison of *MEK1* and *Wis1* function, we overexpressed *MEK1* in wild-type *S. pombe* (Fig. 4). *Wis1* overexpression (as conferred by pREP1-wis1) is lethal to wild-type *S. pombe*, causing cell swelling and lysis [22]. This lethality requires Mcs4, Win1, Wak1 and Sty1 [16,18]. pREP1-MEK1 likewise conferred lethality to the wild-type and the lethality was repressed when *MEK1* expression was decreased by adding thiamine (Fig. 4). pREP1-MEK1 did not confer lethality to Δ*mcs4*, *win1-1* or Δ*wak1* mutants (data not shown), again arguing that these components were required for activating MEK1. Unlike pREP1-Wis1, however, pREP1-MEK1 was lethal in the Δ*sty1* mutant (data not shown), indicating that MEK1 may act on an additional downstream effector(s).

3.3. Co-expression of CTR1 and MEK1 rescues the Δwis1 mutant

The inability of pREP81-MEK1 or pREP82-MEK1 to rescue the Δ*wis1* mutant (Figs. 1 and 5B) allowed us to determine whether CTR1 could enhance MEK1 activity in this system. We found that when we co-transformed the Δ*wis1* mutant with pREP82-MEK1 and pREP41-CTR1 (full length), the cells were able to grow on high osmolarity medium (Fig. 5B). Prior to this, we ascertained that *CTR1* alone could

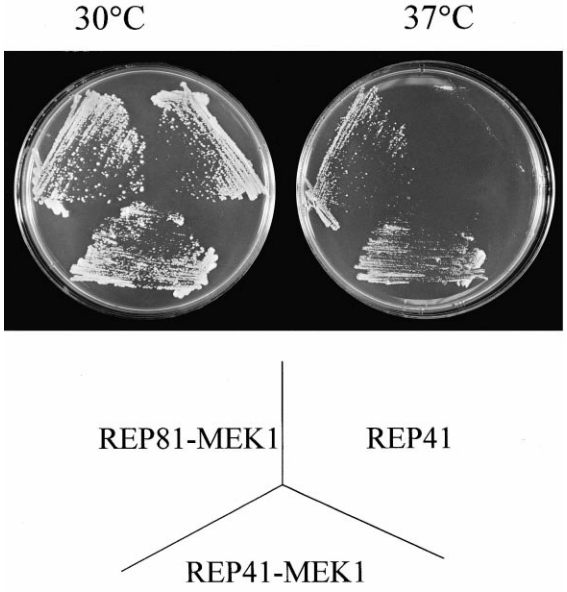

Fig. 3. MEK1 suppresses the temperature sensitivity of the *S. pombe win1-1* mutant. *win1-1* cells (JM1413) were transformed with the indicated plasmid constructs. Transformants were streaked onto YEPD medium and monitored for growth at 30 or 37°C after 3 days. These results paralleled those with *Wis1* (data not shown) and may be due to a Wak1-/Win1-independent pathway controlling Wis1 [18].

not rescue the Δ*mcs4*, *win1-1*, Δ*wak1*, Δ*wis1* or Δ*sty1* mutants (Fig. 5A and data not shown).

Since it has been shown for CTR1 [10] and other MAPKKKs (e.g. [14,15,28–30]) that removal of the non-catalytic N-terminal domain can yield an activated kinase, we tested versions of CTR1 lacking various segments of the N-terminal domain. When these deletion constructs were co-transformed with pREP82-MEK1 into the Δ*wis1* mutant, the cells could grow on high osmolarity medium (Fig. 5B and data not shown). In the absence of MEK1, none of these CTR1 constructs restored growth to Δ*mcs4*, *win1-1*, Δ*wak1*, Δ*wis1* or Δ*sty1* (Fig. 5A and data not shown). The full length *CTR1* construct conferred growth slightly better than did the deletion constructs. Because unpaired constructs were unable to rescue Δ*wis1*, we concluded that CTR1 enhanced the ability of MEK1 to rescue Δ*wis1*.

3.4. Enhancement of MEK1 function by CTR1 does not require CTR1 kinase activity

Further data provided evidence that enhancement of the MEK1 function by CTR1 was not due to CTR1 kinase activity. First, we tested the effect of an Asp-694 to Glu substitution in CTR1, which was expected to cause a reduction in kinase activity. The Asp residue in kinase subdomain VII is highly invariant among serine/threonine protein kinases [31] and the same substitution is encoded by the loss-of-function *ctr1-1* mutant allele, which confers constitutive ethylene responses in *Arabidopsis* [9]. However, this alteration within either the CTR1 full length (Fig. 5) or kinase domain constructs (data not shown) did not affect the ability of CTR1 to enhance the MEK1 function in Δ*wis1*. Second, we found that co-transformation of Δ*wis1* with MEK1 and the CTR1 N-terminal domain (residues 1–550), which lacked the kinase domain, also restored growth on 1.5 M sorbitol (Fig. 5). In fact, the CTR1 N-terminal domain was just as effective as full length CTR1 in this assay. It was therefore unlikely that CTR1 functioned as a protein kinase to activate MEK1 in this heterologous system. Rather, these results pointed to a non-catalytic role for CTR1 in the activation of MEK1.

Finally, we tested whether CTR1 acted alone in enhancing the MEK1 function. If CTR1 acted independently, then, co-expression of CTR1 and MEK1 should have produced the same result in the *win1-1* MAPKKK mutant as in the Δ*wis1* MAPKK mutant. Instead, we found that the *win1-1* mutant that was co-transformed with pREP42-MEK1 and pREP41-CTR1 failed to grow on 1.5 M sorbitol (data not shown),

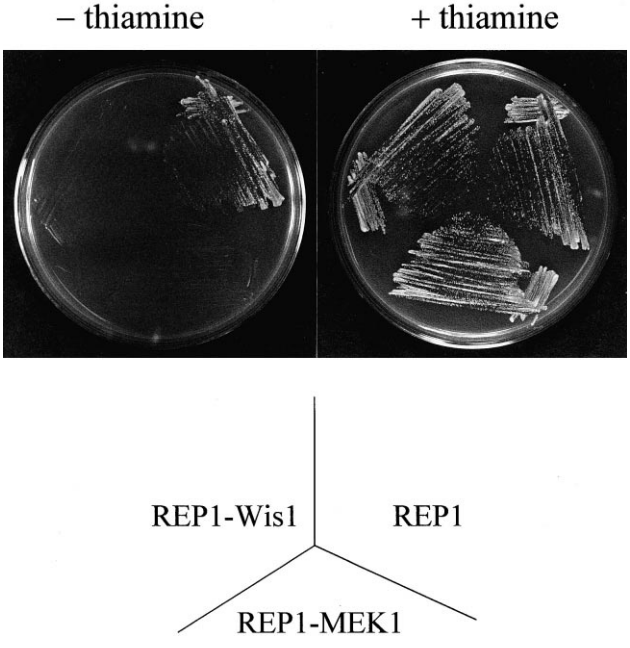

Fig. 4. High expression of *MEK1* is lethal in wild-type *S. pombe* cells. Wild-type cells (JM1059) were transformed with the indicated plasmid constructs. *MEK1* and *Wis1* expression was driven by the thiamine-repressible promoter in pREP1. Transformants were streaked onto EMM medium lacking leucine (to select for plasmids) with or without 16 μM thiamine and monitored for growth after 3 days at 30°C. As shown, results with pREP1-MEK1 paralleled those with pREP1-Wis1.

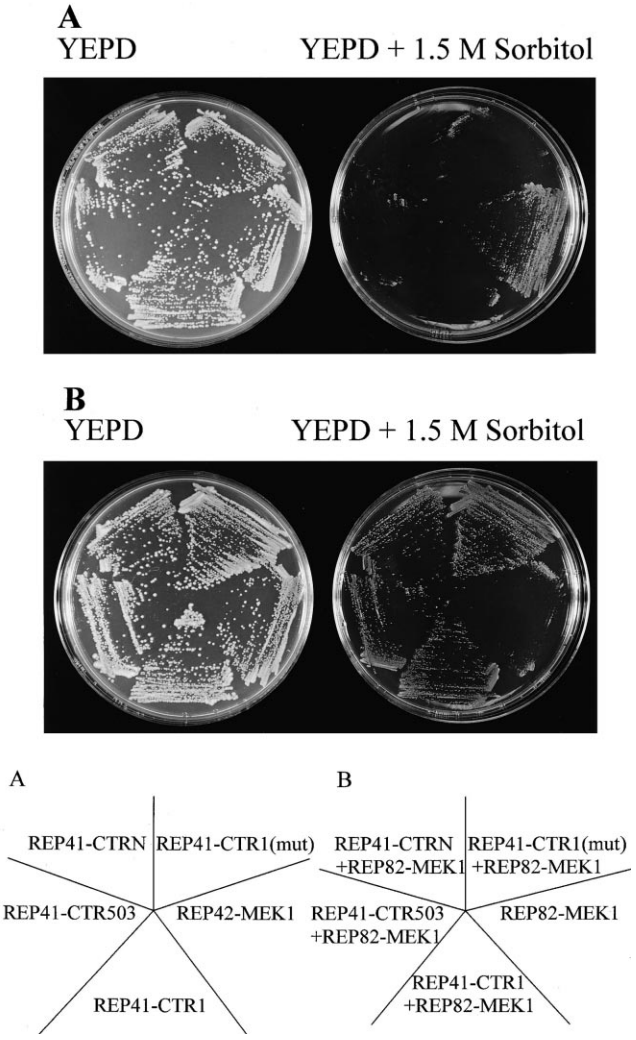

Fig. 5. Functional complementation of the high osmolarity growth defect of Δ*wis1* by co-expression of *Arabidopsis CTR1* and *MEK1*. A: *CTR1* constructs alone do not rescue Δ*wis1*. Δ*wis1* cells (ED1009) were transformed with the indicated plasmid constructs and tested as in Fig. 1. REP41-CTRN contains the CTR1 N-terminal domain (residues 1–550), REP41-CTR1(mut) contains the $Asp^{694} \rightarrow Glu$ substitution in the full length CTR1 and REP41-CTR503 contains the CTR1 kinase domain (residues 503–821). Only REP42-MEK1 conferred growth. B: Co-transformation of *CTR1* and *MEK1* restores growth to Δ*wis1*. Δ*wis1* cells (ED1009) were co-transformed with the indicated plasmid constructs and tested as in Fig. 1. All transformants carrying both plasmids could grow, however, transformants of pREP82-MEK1 alone could not.

indicating that the *Win1* product was required for CTR1 enhancement of the MEK1 function. (We did not test temperature stress in *win1-1* due to the existence of a Win1/Wak1-independent pathway feeding into Wis1/MEK1, as explained above.)

4. Discussion

We have shown that the *Arabidopsis MEK1* gene can substitute for the *S. pombe Wis1* gene in the stress-activated pathway. Functional complementation worked best when *MEK1* was expressed from a strong promoter. We did not observe rescue when *MEK1* was expressed from a weak promoter, perhaps because the MEK1 protein has a lower activity or weaker specificity for Sty1 compared to Wis1. There might also be inefficient utilization of MEK1 by the pathway. Notably, we found that complementation depended upon upstream components of the stress-activated pathway, suggesting that *Arabidopsis MEK1* may encode a functional homolog of *S. pombe Wis1*. This is consistent with the recent report that *MEK1* could rescue the *S. cerevisiae* Δ*pbs2* (MAPKK) mutant of the Ssk2/22-Pbs2-Hog1 osmolarity-response pathway (although it was not determined whether MEK1's function depended upon upstream and downstream components in that pathway) [8]. A separate study showed that co-expression of *MEK1* and the *AMPK4* MAPK gene could rescue the *S. cerevisiae* Δ*mpk1* (MAPK) mutant of the Bck1-Mkk1/2-Mpk1 hypotonic/heat shock-response MAPK pathway [7]. However, rescue occurred even in the absence of Bck1 (MAPKKK), indicating that MEK1 was not activated by Bck1. In addition, we have found that MEK1 only weakly rescues the *S. pombe byr1* (MAPKK) mutant of the mating pheromone-response pathway (Z. Pan and C. Chang, unpublished). The *S. cerevisiae* osmolarity-response pathway and the *S. pombe* stress-response pathway have structural and functional similarities [16]. Stress-activated MAPK pathways ranging from yeast to human are conserved and components have been found to be capable of substituting for each other [1]. For example, mouse JNKK1 can partially substitute for *S. cerevisiae* Pbs2 (MAPKK) [32] and human JNK and p38 can partially substitute for *S. cerevisiae* Hog1 (MAPK) [1,33,34]. Our results, and the results of Mizoguchi et al. [8], indicate that MEK1 can activate the p38 type of MAPK (Sty1 and Hog1). MEK1 may thus act in a functionally homologous pathway in *Arabidopsis*.

When *MEK1* was expressed at lower levels, suppression of Δ*wis1* was achieved by co-expression of *CTR1*, suggesting that CTR1 augmented MEK1 activity. Our data indicated that this result was not caused by direct phosphorylation of MEK1 by CTR1. In fact, the CTR1 N-terminal domain appeared to augment MEK1 activity just as well as the CTR1 kinase domain. Co-expression of *MEK1* and *CTR1* could not suppress the *win1-1* MAPKKK mutant, thus revealing a dependence on *Win1*. Although possible, it is unlikely that Win1 stimulated an unknown catalytic activity of the CTR1 N-terminal domain. It is more likely that CTR1 enhanced Win1 signaling to MEK1 or facilitated the pathway's utilization of MEK1. These latter two explanations implicate an unknown structural role for CTR1. One possibility is that the expression of *CTR1* constructs somehow served to stabilize *MEK1* transcripts or the *MEK1* product, either specifically or non-specifically. Another intriguing possibility is that CTR1 played a scaffolding role. In both the yeast osmolarity-response and animal stress-activated pathways, a MAPKKK and MAPKK, respectively, serve as protein scaffolds for MAPK phosphorylation [30,35–37]. Whatever the mechanism, CTR1 was only required when MEK1 was expressed at low levels. Whether our results reflect a structural function of CTR1 in plants is unknown and may have to await identification of a MAPKK and MAPK in the ethylene-response pathway. Our results show that caution is required when interpreting the results of yeast co-expression studies. Currently, there is no evidence that MEK1 acts downstream of CTR1 in plants. Nevertheless, the heterologous complementation system described here could potentially be utilized to identify regulators of MEK1 activity or to study MEK1 structure or function.

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